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Evaluate the cell toxicity and antiviral analysis of homeopathy medicines in Fish cell lines against *Tilapia Lake Virus* (TiLV)

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Abstract
Tilapia lake virus (TiLV) is a novel virus that causes large scale mortalities in Tilapia farming and is considered as a threat to the global tilapia industry. In the present study, the cell toxicity and antiviral activity of homeopathy medicines against TiLV using SSN1 cell line was investigated. A total of 10 homeopathy medicines including *Belladonna* 200C, *Pyrogenium* 200C, *Influenzinum* 200C, *Crotalus horridus* 200C, *Arsenicum album* 200C, *Hepar sulphur* 200C, *Aconitum napellus* 200C, *Tuberculinum* 200C, *Bothrops lanceolatus* 200C and *Nux vomica* 200C were used at different concentrations *viz*, 100 µl /ml, 250 µl /ml, 500µl /ml and 750µl /ml of medium for screening the antiviral activities against TiLV. Among the 10 medicines, 3 medicines *Crotalus horridus*, *Influenzinum* and *Pyrogenium* inhibited the cytopathic effect caused by TiLV in SSN1 cell line and cell toxicity was occurred at 750µl /ml. Further, these three medicines at a concentration of 100 µl/ml of medium were used for virus neutralization study which showed antiviral activity against two different concentrations of virus ($10^{1.0}TCID_{50}$ ml^{-1} and $10^{6.75}TCID_{50}$ ml^{-1}). In this study, the concentration of homeopathy medicine at 500µl /ml of the medium showed better effect against TiLV in both virus concentrations of $10^{1.0}TCID_{50}$ ml^{-1} and $10^{6.75}TCID_{50}$ ml^{-1} when compared to the control group. Our study has revealed that homeopathy medicines have potent antiviral effects in fish virus in cell culture. The finding suggests that homeopathy medicines have the potential as a therapeutic agent in the treatment of TiLV infection.

Keywords: *Tilapia Lake Virus*, Homeopathy medicines, fish cell lines, cell toxicity and Antiviral effect

Introduction
Tilapia is the second most important fish species for aquaculture next to carps. During 2020, global tilapia production increased by 3.3 percent, reaching 6 million tonnes. China, Ecuador, Egypt, Indonesia and Thailand are the largest tilapia producing countries while the largest importing countries are United States. Nile tilapia (*Oreochromis niloticus*) is the most important candidate species cultured globally among the 100 species of tilapiines. Tilapia is considered as an important candidate species for culture aspect due to their nutritional value, omnivorous diet, tolerance for high-density aquaculture, and relative disease resistance. Although the intensifying culture of tilapia leads to the occurrence of different emerging pathogens Abowei *et al*. (2011) [1].

Initially, tilapias were considered as more resistant to bacterial, parasitic, fungal and viral diseases compared to other cultured species. But Tilapia is susceptible to both bacterial and parasitic diseases including *Streptococcus* sp, *Flavobacterium columnare, Aeromonas hydrophila, Edwarsiella tarda, Ichthyophitirius multifillis, Tricodhina* sp, and *Gyrodactylus niloticus* Klesius *et al*., (2008) [13]. There is no report viral disease in Tilapia until 2009. During the summer of 2009 in Israel, there was enormous amount of mortality observed in both wild and farmed hybrid tilapia (*O. niloticus* × *O. aureus*) and the etiological agent was subsequently identified in 2013 as Tilapia Lake Virus (TiLV) Eyngor *et al*. (2014) [8]. After that, the virus has been reported in various countries including Israel, Thailand, Ecuador, Colombia, Egypt, Kerala, West Bengal and Tamilnadu in India Behera *et al*. (2018) [4]; Dong *et al*. (2017) [7]; Eyngor *et al*. (2014) [8]; Ferguson *et al*. (2014) [10]; Saranya *et al*. (2020) [31]. Several fish species including hybrid tilapia (*Oreochromis nil*oticus × *O. aureus*), Nile tilapia

(*O. niloticus*), grey tilapia (*O. niloticus* × *O. aureus*), red tilapia (*Oreochromis* sp.), Mozambique tilapia (*O. mossambicus*) Amal *et al*. (2018) [2]; Eyngor *et al*., (2014) [8]; Fathi *et al*. (2017) [9]; Ferguson *et al*. (2014) [10]; Mugimba *et al*. (2018) [23]; Surachetpong *et al*. (2017) [33]; Waiyamitra *et al*. (2021) [36], giant gourami (*Osphronemus goramy*) Chiamkunakorn *et al*. (2019) [6] ; Jaemwimol *et al*. (2018) [11]. ornamental African cichlids (*Aulonocara* spp) Yamkasem *et al*. (2021) [39] have been found to infect by TiLV.

TiLV disease is an emerging and transboundary disease of tilapia culture, causing mortality up to 90% globally in farmed tilapia over the last 4-5 years Eyngor *et al*. (2014) [8]. The virus is a negative-sense, single-stranded RNA virus (-ssRNA) made of an icosahedron envelope with a genome length of 10,323 kb and 55-100 nm diameter Eyngor *et al*. (2014) [8]. It is an orthomyxo-like virus and the only member of the genus Tilapinevirus in the family Amnoonviridae Eyngor *et al*. (2014) [8]; Bacharach *et al*. (2016) [3]. This virus infected fish could show symptoms that include anorexia, abnormal swimming, severe anemia, exophthalmia, skin erosion and congestion, scale protrusion, and abdominal swelling.

The occurrence and spreading of disease can be avoided by following the proper health management approaches. Some experimental vaccines have been developed for TiLV that are heat-killed and formalin-killed vaccine Mai *et al*. (2022) [17], DNA vaccine Yu *et al*. (2021) [40], VP20-based vaccine Zeng *et al*. (2021) [41], inactivated vaccine containing montanide adjuvant Zeng *et al*., (2021) [42], but these are not yet commercialized for aquaculture use. Antibiotics and other chemotherapeutics have been used to control fish and shellfish diseases but could result in the development of drug-resistant pathogens and environmental threats Rico *et al*. (2012) [29]. Homeopathy medicine can be used as an alternative option to control TiLV infection. Therefore, the present study is intended to evaluate the antiviral effect of different homeopathy medicines against TiLV-2 in cell culture.

Materials and Methods

Virus strain and Cell line

The virus TiLV isolated from the infected tilapia fish was used for the study. The SSN1 cell line derived from snakehead fish in the Department of Fish Pathology and Health Management of FCRI, Thoothukudi was used for virus isolation and propagation. The virus titer ($TCID_{50}$/ml) was determined by end-point dilution assay using 96 well plates and calculated according to the method of Spearman-Karber Lei *et al.* (2021) [14].

Homeopathy medicines

In the present study, 10 homeopathy medicines including *Belladonna* 200C, *Pyrogenium* 200C, *Influenzinum* 200C, *Crotalus horridus* 200C, *Arsenicum album* 200C, *Hepar sulphur* 200C, *Aconitum napellus* 200C, *Tuberculinum* 200C, *Bothrops lanceolatus* 200C and *Nux vomica* 200C were tested for their antiviral effect against TiLV infection in cell culture. These homeopathy medicines were purchased from Dhivyam Medicals, Madurai.

Determination of the cytotoxicity of the homeopathy medicines

The cytotoxicity of the homeopathy medicines was determined in SSN1 cell cultures by cell viability. For this, medicines were added to the cell cultures in concentrations of 100, 300, 500 µl/ml and 750 µl/ml incubated for 5 days. The viability of the cells was determined by staining with a trypan blue solution (0.5%).

Treatment with homeopathy medicine

Experiment - 1

The assay was performed in the full monolayer of SSN1cell line developed in cell culture plastic flask (25 cm^2) (Thermo, Korea). When the monolayer became sufficiently confluent, the TiLV was inoculated with 100, 300 and 500 µl of the stock (10^1TCID_{50}/ml and $10^{6.75}$ $TCID_{50}$/ml) and incubated for 15 - 20 mins at room temperature. Followed by incubation, the virus solution was removed and homeopathy medicine was added with a concentration of 500 µl/ml of medium. After 72h of incubation, the result was observed Nefedchenko *et al.* (2015) [26].

Experiment - 2

Neutralisation test was carried out by titrating a constant quantity of virus with the homeopathy medicines in 96 well plate after developing the full monolayer of SSN1 cells. Maintenance medium (L-15 medium) amounting to 90 µl was added into each well except column one. TiLV preparation (100 µl) was added to the first column as per the protocol in duplicate. The virus as serially double diluted by transferring 10 µl across the plate. A back titration of the TiLV preparation was also carried out simultaneously in the same titration plate to confirm the amount of virus used in the assay. A 100 µl of homeopathy medicine was added to each well in diluted virus bearing rows and maintenance medium was added at 100 µl in to all wells having TiLV preparation. Finally, the plate was incubated at 27 ˚C and CPE development was recorded over 5- 10 days.

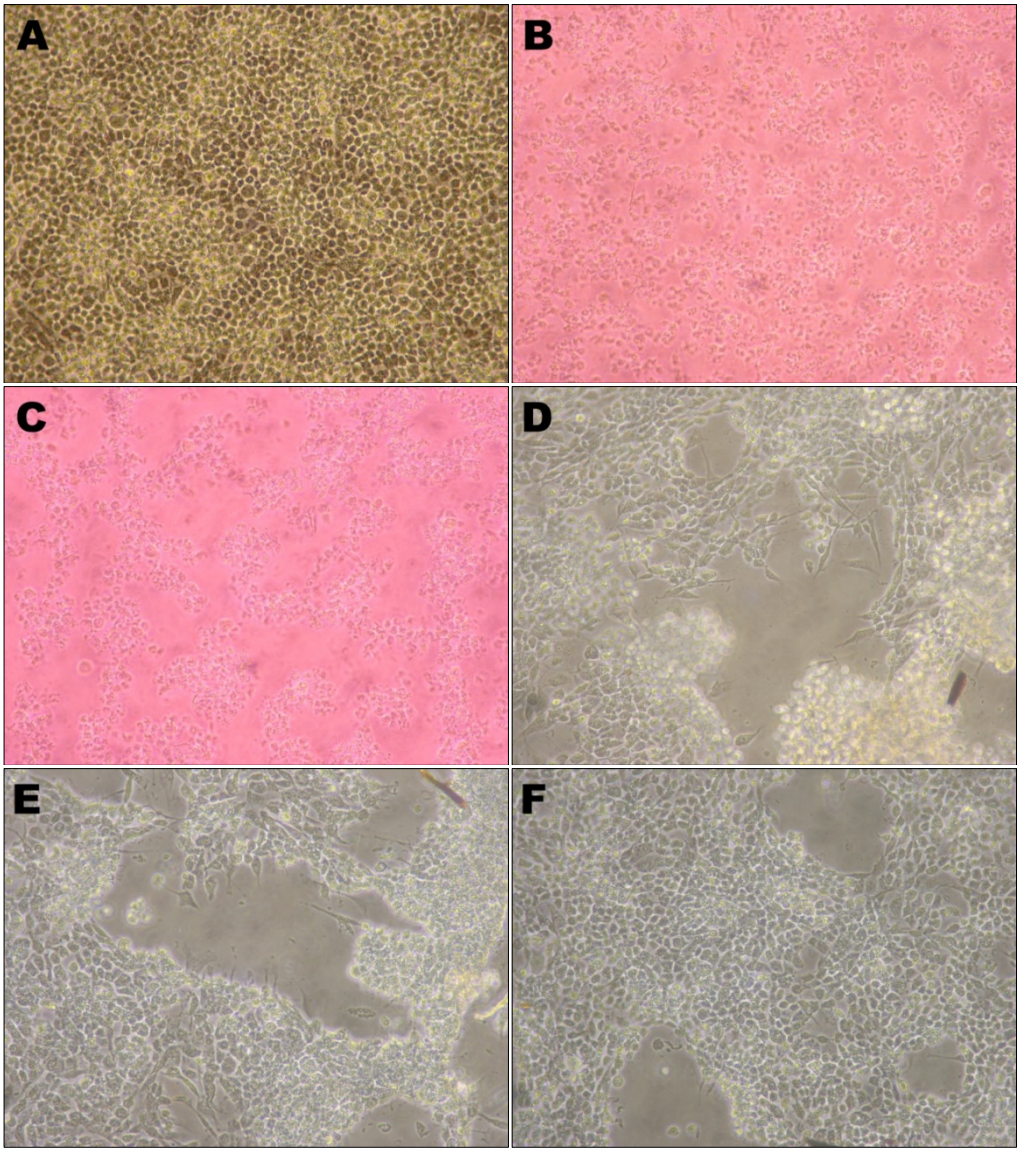
Results

A total of ten remedies were tested for their toxicity to SSN1 cell cultures. All the medicines exhibited cell toxicity only at 750 µl/ml by causing morphological changes and increasing dead cells. Further, these homeopathy medicines were screened for detecting the antiviral effect against TiLV using the SSN1 cell line. Out of ten, three homeopathy medicines such as *Crotalus horridus* 200C, *Influenzinum* 200C and *Pyrogenium* 200C showed the antiviral effect against TiLV by reducing the cytopathic effect as shown in figure 2. 100 µl/ml, 300 µl/ml, 500 µl/ml concentration of homeopathy medicines was evaluated and out of these three concentrations, 500 µl/ml of homeopathy medicines shows antiviral activity. The potential antiviral effect of *Crotalus horridus* 200C, *Influenzinum* 200C, *Pyrogenium* 200C were found as 100%, 95% and 70% respectively (figure 3, 4, 5). Among these three medicines, *Crotalus horridus* 200C has shown the highest (100%) potential against TiLV. Upon infection, the virus inoculated SSN1 cell line monolayer exhibited typical cytopathic effect with the appearance of small foci with cell lysis and rounding up of cells at the edge of foci. No cytopathic effect was found in mock inoculated.

In the second experiment, the infectivity of TiLV concentrated to 10^1TCID_{50} and $10^{6.75}TCID_{50}$ were completely neutralized by *Crotalus horridus* 200C and *Influenzinum* 200C. *Pyrogenium* 200C was found to be completely neutralized the infectivity of TiLV concentrated to 10^1TCID_{50} while other concentration shows the reduction in the infectivity from $10^{6.75}$ to $10^{3.75}TCID_{50}$/ml during 7 days observation period. Parallel to homeopathy medicine neutralization, the infectivity of SRDV in SSN1 cell line

was found to be $10^{6.75}$ $TCID_{50}$/ml, while no CPE was noticed in the control cell line which is mock inoculated.

Fig. 1: Tilapia fish infected by TiLV



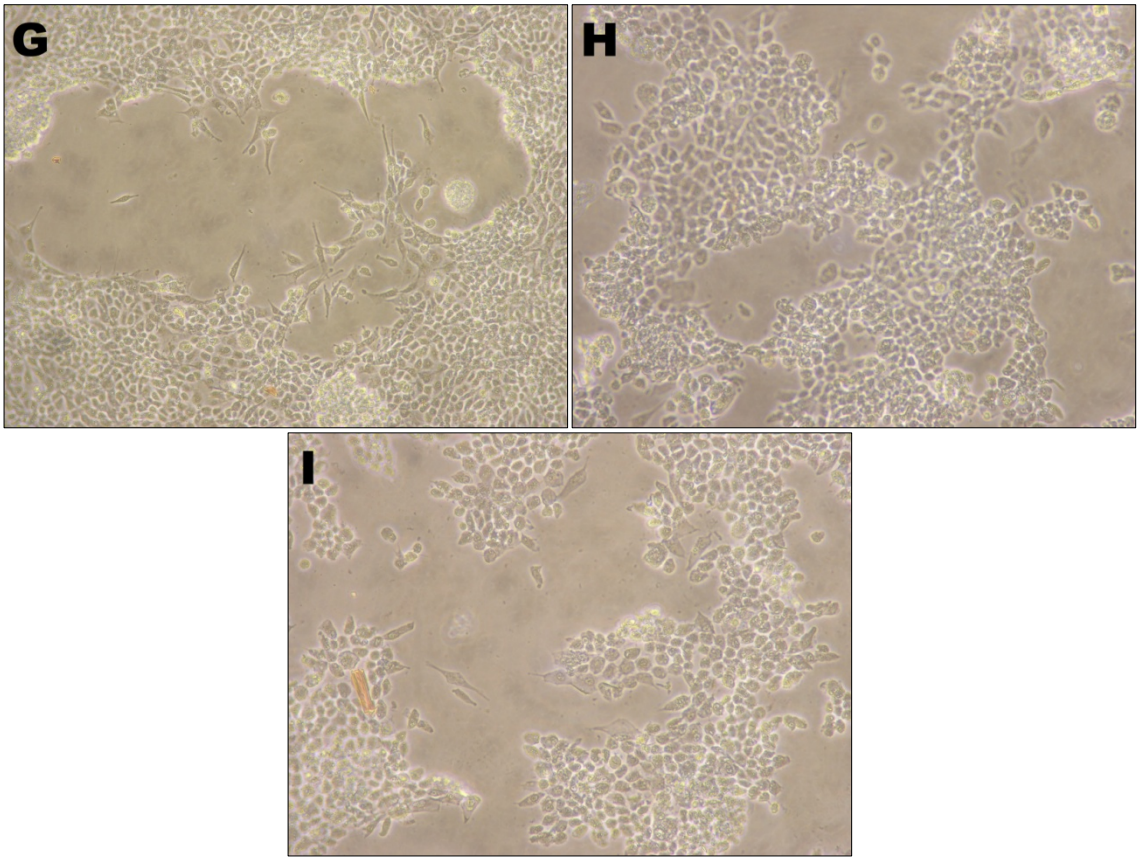


Fig 2: Cytopathic effect induced by TiLV in SSN1 cells. (A) Uninfected control SSN1 cells (B) TiLV (10^{1}TCID$_{50}$/ml) infected cells (C) TiLV ($10^{6.75}$TCID$_{50}$/ml) infected cells(D) TiLV (10^{1}TCID$_{50}$/ml) infected SSN1 cells treated with *Crotalus horridus* 200C (E) TiLV ($10^{6.75}$TCID$_{50}$/ml) infected SSN1 cells treated with *Crotalus horridus* 200C (F) TiLV(10^{1}TCID$_{50}$/ml) infected SSN1 cells treated with *Influenzinum* 200C (G) TiLV ($10^{6.75}$TCID$_{50}$/ml) infected SSN1 cells treated with *Influenzinum* 200C (H) TiLV (10^{1}TCID$_{50}$/ml) infected SSN1 cells treated with Pyrogenium 200C (I) TiLV ($10^{6.75}$TCID$_{50}$/ml) infected SSN1 cells treated with Pyrogenium 200C.

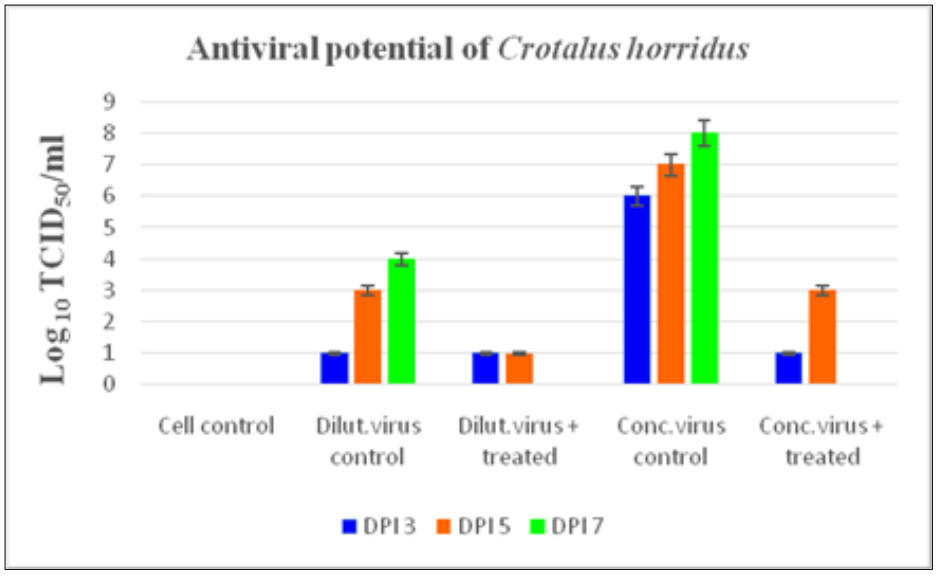


Fig 3: Antiviral activity of homeopathy medicine *Crotalus horridus*

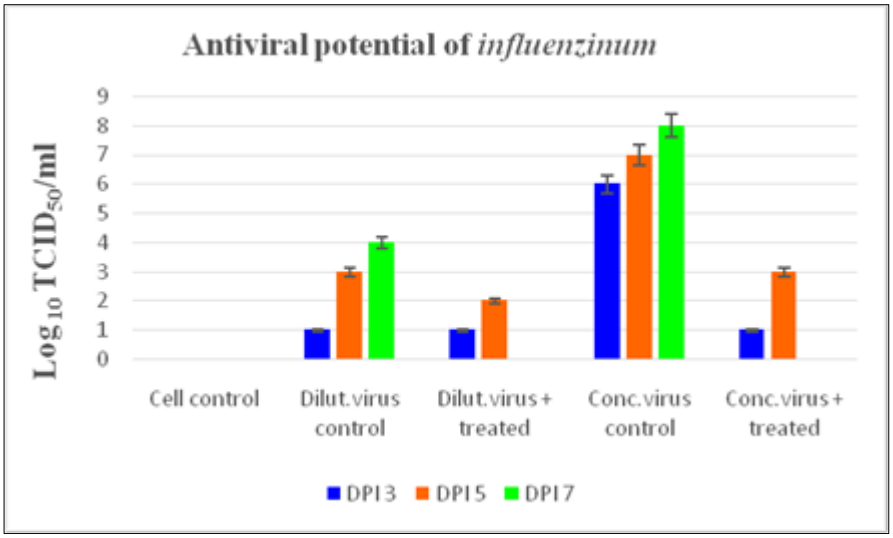


Fig 4: Antiviral activity of homeopathy medicine *Influenzinum*

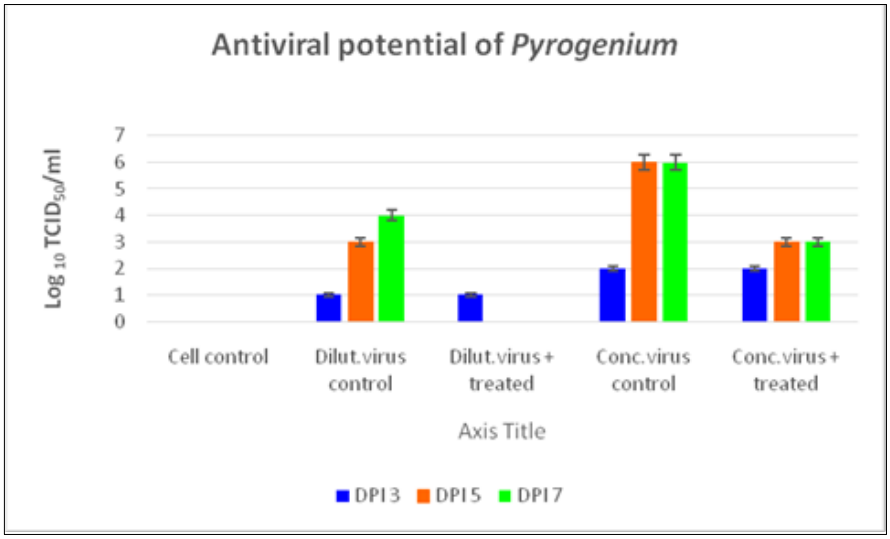


Fig 5: Antiviral activity of homeopathy medicine *Pyrogenium*

Discussion

During the past few years, the TiLV outbreak has caused a severe loss in tilapia farming industry and resulted in greater risk in tilapia culture Zhou *et al*. (2017) [43]. To date, there is no commercial vaccine available to prevent TiLV. Hence, alternative studies on therapeutics or compounds to control TiLV outbreaks, especially antiviral agents, are urgently needed Lertwanakarn *et al*. (2021) [15]. Besides, the use of antibiotics is associated with many problems that are spread of antibiotic residues in the culture environment, increasing the rates of antibiotic resistance in aquatic pathogens and, critically, transferring that resistance to human pathogens Pepi *et al*. (2021) [28].

Homeopathic medicines are natural substances of animal, plant or mineral origin Ortiz-Cornejo *et al*. (2017) [27]. that could activate specific sensibility mechanisms in living organisms Mazón-Suástegui *et al*. (2018) [21]. Homeopathy medicine is useful in both human and veterinary treatment Mayer *et al*. (2016) [19]. It can also be used to heal fish because it works with all living organisms. Homeopathy medicines can be used to treat TiLV infection as it has been used with success in aquaculture Mazón-Suástegui *et al*. (2017) [20]. The intramuscular injections of homeopathic drug Heaper Sulphur and Arnica spray have shown encouraging results in curing the ulcerative syndrome in fish caused by fungal infection Mitra *et al*. (1991) [22]. Homeopathic treatments have the potential as an alternative for the control of *V. parahaemolyticus* and its associated diseases in shrimp farming. Cell culture is a very versatile tool which can be used as a model system to study the interaction between cells and viruses and to study the effect of drugs. In the present study, the antiviral effects of different homeopathy medicines were investigated against TiLV using SSN1 cell line.

Previous studies have found that TiLV infection can lead to the formation cytopathic effect and massive cell death within 3-7 days in various fish cell lines Liamnimitr *et al*. (2018) [16]; Nanthini *et al*. (2019) [24]; Thangaraj *et al*. (2018) [34]; Wang *et al*. (2018) [37]; Yadav *et al*. (2021) [38]; Lertwanakarn *et al*. (2021) [15]. In the present study, TiLV inoculated SSN1 cell line monolayer exhibited a typical cytopathic effect with the appearance of small foci with complete cell lysis within 4 days. In the previous study, homeopathy medicines such as Engystol and Echinacea com have found to be exhibited toxicity at 500 µl/ml in MDBK and KST cell cultures Nefedchenko *et al*. (2015) [26]. In this study, all the medicines exhibited toxicity to SSN1 cells only at 750 µl/ml.

In other studies, ribavirin is a synthetic nucleoside

analog inhibited virus replication and reduced ISAV viral loads and CPE formation in fish cell lines. Similarly, ribavirin reduced CPE formation and viral loads in cells incubated with ISAV, IHNV, IPNV and VHSV Rivas-Aravena *et al*. (2011) [30]; Marroqui *et al*. (2007) [18]; Kim *et al*. (2011) [12]. In this study, ten homeopathy medicines were tested for their antiviral effects against TiLV infection in which three medicines significantly reduced TiLV viral load and formation of CPE in SSN1 cell line. Homeopathy remedies such as *Crotalus horridus* 200C, *Influenzinum* 200C and *Pyrogenium* 200C showed antiviral effect against TiLV at the concentration of 500 µl/ml of medium. *Crotalus horridus* 200C and *Influenzinum* 200C remedies exhibited higher cell survival and complete reduction in CPE formation while the *Pyrogenium* 200C shows slightly less antiviral effect against TiLV-2 in cell culture.

In the virus neutralization test, three remedies *Crotalus horridus* 200C, *Influenzinum* 200C and *Pyrogenium* 200C exhibited complete neutralization of TiLV infectivity in the SSN1 cells within 7 days. *Crotalus horridus* has been used as remedy for human and veterinary animals. *Crotalus horridus* has been used against babesiosis and canine ehrlichiosis diseases in dogs Chaudhuri *et al*. (2007) [5] and Tungnunga *et al*. (2016) [35]. It is also used for increasing the platelet count against dengue virus in humans Nayak *et al*. (2019) [25]. Homeopathy remedies can also be used for stimulating digestive processes and detoxicating (*Nux vomica* and *Chelidonium*) and immune-stimulant (*Echinacea purpurea* and *Lycopodium*) Sarubbi *et al*. (2012) [32].

Conclusion

This study presents the first evidence of the application of homeopathy medicine against TiLV in fish cells in vitro. We demonstrated that homeopathy medicine reduces CPE formation, inhibits TiLV viral replication and improves cell survival. On the whole, our study demonstrates the potential of homeopathy remedies against TiLV. This new information could be used in future research on the efficiency of homeopathy remedies and pathogenesis and replication mechanisms of this novel virus. Further in vivo research is required to demonstrate the efficacy of homeopathy remedies in tilapia during experimental infection TiLV. This study will pave the way for the further use of homeopathy medicine as an antiviral agent in the aquaculture sector.

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Authors' Contribution

Rakesh P: Formal analysis and writing original draft of the manuscript; Sivasankar P & Chrisolite B: conceptualized, methodology, designed and supervision of the study; Magesh Kumar P, Mohamed Mansoor M & Selvamagheswaran M: reviewed the draft of the manuscript; Ramesh P: data analysis and interpretation.

Ethical approval: No animals were harmed during the entire research.

Conflict of interest: The authors have declared no conflict of interest.

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